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Quantitative and qualitative studies of antibody–induced mesangial cell damage in the rat

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Quantitative and qualitative studies of antibody–induced mesangial cell damage in the rat. Intravenous administration of heterologous anti-rat thymocyte serum (ATS), which reacts with a Thy-1-like antigen present on rat glomerular mesangial cells, caused lytic (1 hr to 2 days), hypercellular (4 to 14 days), and sclerotic (2 to 3 months) mesangial lesions in Lewis rats. The normal control of 48.6 ± 7.9 (mean ± SD) glomerular nuclei on histologic section decreased significantly ($P < 0.001$) to 39.8 ± 6.1, 37.4 ± 6.0, and 38.9 ± 6.8 at one hour, four hours and two days after ATS administration, respectively. Thereafter glomerular nuclei increased to 54.7 ± 11.5 ($P < 0.05$) at four days, 62.5 ± 9.6 ($P < 0.001$) at one week and 64.1 ± 14.2 ($P < 0.001$) at two weeks, and normalized ($P > 0.05$) to 49.4 ± 8.9 at one month and 50.6 ± 9.0 at three months. By electron microscopy, glomerular damage in the lytic stage was restricted to mesangial cells and was manifested as hydropic degeneration or lysis. Rabbit IgG and rat C3 were found in the mesangium one hour after injection; they decreased at two days and were negligible at four days. By paired label isotope study, 11.6 μg of antibody bound per 7.6×10^4 glomeruli at one hour was needed to induce mesangial cell degeneration. No or only minimal changes in proteinuria and in serum creatinine were observed with the dosage used in this rat strain. The glomerular uptake of aggregated human gammaglobulin, aggregated bovine serum albumin or preformed immune complexes in ATS-treated rats exceeded that in controls by 13.9, 14.6, and 4.1 times, respectively, at four hours after aggregate administration, but not at 24 hours, in quantitative studies of mesangial function. The selective, antibody–induced glomerular cell–injury of this new model contrasts with that of established models of glomerulonephritis in which immune deposit formation and mediator activation cause relatively–nonspecific glomerular inflammation.

The mesangial cell is thought to play a central role in immune deposit forms of glomerular injury as well as in the normal physiology of glomeruli [1]. In conditions such as IgA nephropathy in humans, mesangial deposits are prominent and, rarely, anti-mesangial antibody reactions have been suggested [2]. The mesangium may help clear aggregates from the glomerular capillary lumen by the phagocytic function of its cells and by transport through its channels [1, 3, 4]. Mesangial cells also produce a number of products including enzymes, arachidonic acid metabolites, and interleukin-1-like materials that could influence the course of glomerular injury [5–8]. The mesangial cell has angiotensin II receptors and can respond to this stimulus [9], suggesting a role for the cell in glomerular hemodynamics. However, the mesangial cell has been hard to study in vivo since there has been no easy way to selectively injure the cell other than possibly with certain snake venoms [10, 11].

Recently, a Thy-1-like antigen was demonstrated in the rat glomerulus by immunofluorescence microscopy using monoclonal anti-Thy-1.1 antibody or heterologous anti-rat thymocyte antibody [12–14]. The Thy-1-like antigen was localized on the surfaces of mesangial cells by immunoelectron microscopy [15, 16]. The induction of histological glomerular lesions by intravenous administration of heterologous anti-thymocyte serum has been inconsistent. Bagchus et al found antibody binding to the mesangium and the glomerular basement membrane in rats that received heterologous anti-thymocyte antibodies; however, no light microscopic glomerular changes were observed [17]. In contrast, Yamamoto et al found disappearance and destruction of mesangial cells two days after the administration of anti-rat thymocyte serum [16, 18]. Mesangial lesions have also been induced with intravenous administration of monoclonal anti-thymocyte antibodies [19, 20]. The mesangial cell injury appears to occur independently of antiglomerular, basement membrane antibody–mechanisms, which sometimes have been associated with anti-lymphocyte sera [21].

To help clarify the concept of direct antibody-induced glomerular cell injury and to study the function of mesangial cells in macromolecular clearance, we analyzed sequential changes in ATS-induced glomerular lesions, quantitative glomerular binding of the antibody, and alterations of glomerular handling of macromolecules. We found that in comparison to standard immunologic models of glomerular injury only small amounts of bound antibody were needed to induce the selective degeneration and destruction of mesangial cells, subsequent mesangial hypercellularity, and altered glomerular uptake of macromolecular aggregates and immune complexes.

Methods

Preparation and characterization of ATS

ATS was produced in New Zealand White rabbits by immunization with 1×10^8 Lewis rat thymocytes in complete Freund's adjuvant followed by 1×10^6 cells intravenously (i.v.) two and four weeks later [14]. Sera obtained one week after the last injection were heat inactivated (56°C for 30 min) and absorbed three times with packed Lewis–rat erythrocytes and three times with crude Lewis rat, liver membrane fractions [22].

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The tissue reactivity of the ATS was studied by indirect immunofluorescence using dilutions [1:25 to 1:10,000 in phosphate buffered saline (PBS), pH 7.2] on acetone–fixed cryostat sections of normal rat kidney and thymus. To detect cross–reactive antigens in the kidney, thymus, and other organs, ATS was absorbed with rat thymocytes or lyophilized tissue powder prepared from snap–frozen Lewis rat brain, kidney, liver, lung, spleen and thymus; the residual reactivity was compared using indirect immunofluorescence on Lewis rat kidney and thymus sections.

To better define the glomerular antigenic determinants reactive with the ATS, monoclonal antibodies (200 μg/ml) were used to block the indirect immunofluorescence reactions to the ATS. OX7 reactive with rat Thy-1.1 and OX2 reactive with rat thymus glycoprotein (Accurate Chemicals and Scientific Co., Westbury, New York, USA) were used to pretreat tissue sections for 30 minutes at room temperature. After washing with PBS, the sections were reacted with dilutions of ATS and stained with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG antibody (Cooper Biomedical Inc., Malvern, Pennsylvania, USA) that had been absorbed to remove anti-mouse IgG reactivity. The indirect immunofluorescence studies were done as described above.

Induction of glomerular lesions with ATS

Female Lewis rats (6 to 7 weeks old) were used for i.v. administration of ATS (0.05 to 2.0 ml/100 g body wt). Their kidneys were then removed by unilateral nephrectomy and/or at sacrifice at multiple time points between one hour and three months later; three or more rats were used at each time point. No histologic or functional changes were detected in the kidneys from rats that received similar amounts of normal rabbit serum.

The kidneys were examined by light, immunofluorescence and electron microscopy. For light microscopy, the kidney tissues were fixed in Bouin's solution and sections were stained with periodic acid Schiff. To compare the glomerular cell number semiquantitatively, nuclei were counted in over 30 glomeruli of 80 to 100 μm diameter from each kidney. The nuclei of polymorphonuclear leukocytes (PMNs) were counted separately and fragmented debris of degenerating nuclei were omitted. For immunofluorescence microscopy, renal tisues were snap–frozen in liquid nitrogen and cryostat sections were fixed in cold acetone for five minutes for staining with FITC-labeled goat anti-rabbit IgG, rabbit anti-rat C3 and rabbit anti-rat IgG (Cooper Biomedical Inc., Malvern, Pennsylvania, USA). The intensity of glomerular staining was arbitrarily graded from − to +++.

Small pieces of kidneys were fixed with modified Karnovsky's fixative at 4°C overnight and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate for electron microscopy.

Urinary protein excretion was measured by the sulfosalicylic acid method [23] in 24-hour urine samples collected daily in the first two weeks after the ATS injection and weekly in the following three months. Serum creatinine was determined by the method of Heinegard and Tiderstrom [24].

Quantitation of ATS binding

To establish the kinetics of ATS binding to glomeruli, gammaglobulin fractions (precipitated by 50% saturated ammonium sulfate) of ATS and normal rabbit serum (NRS) were labeled with ^{125}I and ^{131}I, respectively [25], for paired label isotope studies [21]. Mixtures containing 100 μg of each protein were given i.v., and groups of four rats were sacrificed 15 minutes, 1, 6, 24, and 96 hours later. At sacrifice, the rats were perfused with PBS through the right ventricle, and the brains, hearts, thymuses, lungs, livers, spleens and kidneys were removed, weighed, and the radioactivity determined. Protein–bound counts in the plasma were determined by precipitation in a final concentration of 10% trichloroacetic acid. Glomeruli were prepared from the kidney cortex by passage sequentially through #60- and #100-mesh screens. The radioactivity in the glomeruli in suspension retained on a #200-mesh screen was determined and their number estimated by counting 20 μl aliquots under a light microscope. The glomeruli were then washed twice with distilled water, frozen, lyophilized, and weighed. After the radioactivity was counted, the other organs were also frozen in liquid nitrogen, lyophilized, and weighed. The specific uptake was calculated as follows: ^{125}I-specific organ count = ^{125}I cpm per organ-[^{131}I cpm per organ × ^{125}I cpm per ml plasma/ ^{131}I cpm per ml plasma]. Specific organ uptake = specific organ count/specific activity. Student's *t*-test for unpaired data was used for statistical analysis.

The amount of glomerular–bound ATS necessary to induce morphologically detectable glomerular injury was quantitated. A 1/50 volume of ^{125}I-ATS and ^{131}I-NRS gammaglobulin (6.2 mg rabbit IgG/ml each) was added to unlabeled ATS also containing 6.2 mg/ml of rabbit IgG, as estimated by radial immunodiffusion. Groups of two rats each received 0.05 to 5.0 ml of the mixture per 100 g body weight. They were sacrificed one hour after the injection, and the amount of rabbit IgG bound to their isolated glomeruli was calculated as above.

Effects of ATS on the quantitative mesangial uptake of macromolecular aggregates and immune complexes

Preparation of macromolecular aggregates. Heat–aggregated human gammaglobulin (AHGG) was prepared as described previously [26]. Human gammaglobulin (HGG, 20 mg/ml in PBS) was mixed with ^{125}I-labeled HGG at a concentration of 20 μg/ml, heated to 63°C for 15 minutes, and cooled rapidly in an ice bath. A 1/2.5 volume of 2.18 M sodium sulphate was added and the mixture was stirred for 30 minutes at 4°C. The resulting precipitate (3000 × g for 30 min) was resuspended and dialyzed against PBS. The dialyzed aggregated HGG was centrifuged at 100,000 × g for 90 minutes, and the pellet was resuspended in the cold PBS and centrifuged at 5,000 × g for 30 minutes to remove insoluble aggregates. The concentration of protein was adjusted to 50 mg/ml of AHGG, including 50 μg/ml of ^{125}I-labeled AHGG and ^{131}I-labeled unaggregated HGG, for use in quantitative, paired label experiments. The soluble AHGG was characterized by sucrose density gradient (10% to 60% wt/vol) ultracentrifugation.

To prepare heat–aggregated bovine serum albumin (ABSA), bovine serum albumin (BSA) was dissolved in 0.15 N NaCl, pH 7.0, at a concentration of 30 mg/ml containing ^{125}I-labeled BSA (30 μg/ml). The solution was heated at 70°C for 20 minutes

followed with shaking, and at 79°C for an additional five minutes. The solution was rapidly cooled with tap water and 0.1 N HCl solution was added to lower the pH to 5.2 to obtain an insoluble precipitate. The mixture was centrifuged at 1000 × g for 10 minutes and the precipitate was washed four times with 0.15 N NaCl, pH 5.2. The precipitate was then dissolved in PBS, kept overnight at 4°C, and centrifuged at 2000 × g for 20 minutes to remove insoluble aggregates. The concentration of ABSA was adjusted to contain 25 mg/ml including 25 μg/ml of ^{125}I-labeled ABSA and ^{131}I-labeled unaggregated BSA each.

Soluble immune complexes of goat, anti-human IgG antibody and HGG (20 mg/ml in saline) containing ^{125}I-HGG at a concentration of 20 μg/ml were prepared. The goat antiserum was decomplemented (56°C for 30 min), and the antibody content was determined by quantitative precipitin analysis [27]; specific antibody was present at a concentration of 11.5 mg/ml. Soluble complexes were prepared by adding the equivalence amount of HGG (20 mg/ml containing 20 μg/ml of ^{125}I-HGG) in saline to the antiserum, mixing thoroughly and incubating the mixture at 37°C for one hour and 4°C overnight. The resulting precipitate was washed with cold saline three times and resuspended in a volume of HGG solution sufficient to give 20 times antigen excess. The pH was lowered to 2.4 by dropwise addition of 1 N HCl at 4°C. After complete dissolution of the precipitate, the pH was readjusted to 7.0 by adding 1 N NaOH at 4°C. The solution was centrifuged at 2000 × g for 20 minutes to discard the insoluble aggregates. ^{131}I-goat gammaglobulin (GGG), prepared from normal goat serum by 50% ammonium sulfate precipitation, was added to the solution in a concentration equivalent to the ^{125}I-HGG. The resulting solution contained 20 mg/ml of HGG and 20 μg/ml each of ^{125}I-HGG and ^{131}I-GGG. The proportion of HGG formed immune complex was examined by precipitation of ^{125}I-HGG in 3.5% polyethylene glycol, as described previously [28].

Assessment of mesangial function. Mesangial damage was induced by administration of 0.5 ml of ATS (or control NRS) per 100 g body weight to 6-week old, female Lewis rats. Twenty–four hours after the ATS or NRS injection, the rats received 50 mg of AHGG containing ^{125}I-AHGG and ^{131}I-HGG per 100 g body weight i.v. Four rats each receiving ATS or NRS were sacrificed at one, four and 24 hours after administration of the aggregate. Systemic clearance of the AHGG was measured in rats destined for sacrifice 24 hours after the AHGG administration. The uptake of AHGG in organs or glomeruli was measured by the paired label technique described above. The kidneys were also examined by immunofluorescence microscopy using FITC-goat anti-human IgG (Cooper Biomedical Inc., Malvern, Pennsylvania, USA).

For ultrastructural localization of aggregate uptake by immunoelectron microscopy, small slices of renal cortex were fixed with 2% paraformaldehyde, 0.2% glutaraldehyde, and 0.2% picric acid in 0.1 M cacodylate buffer, pH 7.2, at 4°C overnight, then postfixed with 0.05% osmium tetroxide in the same buffer at room temperature for 15 minutes. The fixed pieces of tissue were partially dehydrated through 30, 50, 70, 80% ethanol series, embedded in L.R. White resin (London Resin Co., Hampshire, UK), and cured at 50°C for 48 hours. Thin sections were mounted on uncoated #200-mesh nickel grids. Colloidal gold granules ranging from 6 to 8 nm in diameter were produced by the reduction of chloroauric acid with sodium borohydride and complexed to goat, anti-rabbit IgG antibody (Dr. C.M. Chang, Scripps Clinic and Research Foundation, La Jolla, California, USA). The sections were first incubated with rabbit anti-human IgG (40 μg/ml) in 0.05 M Tris-HCl buffered saline, pH 7.6, containing 0.3% BSA, followed by gold–goat anti-rabbit IgG for one hour at room temperature. After immunolabeling, the sections were further fixed in 1% osmium tetroxide for two minutes and stained with aqueous uranyl acetate.

To further examine the effect of ATS on the glomerular accumulation of AHGG, the dose of AHGG (50, 25, 10 or 2.5 mg) and the time intervals (simultaneously, 4 hr, 24 hr, 3 days or 7 days) between ATS and AHGG administration were varied in groups of three or more rats each. All animals were sacrificed one hour after the AHGG injection for measurement of the aggregate's uptake by glomeruli.

To study the effect of ATS on the glomerular localization of ABSA, two groups of eight rats each were given either ATS or NRS at a dose of 0.5 ml/100 g body weight. Twenty–four hours later, they were injected with 25 mg of ABSA containing 25 μg/ml of ^{125}I-ABSA and ^{131}I-BSA per 100 g body weight. Four rats from each group were sacrificed four and 24 hours after the ABSA administration.

To compare the glomerular uptake of AHGG and ABSA with that of preformed immune complexes, two groups of 10 rats received either ATS (0.5 ml/100 g body weight) or NRS i.v. 24 hours before administration of the immune complex preparation (× 20 antigen excess) containing 40 mg of HGG per each 100 g body weight. Five rats from the ATS- and NRS-treated groups were sacrificed at four and 24 hours. The specific uptake was expressed as μg of AHGG, ABSA, and HGG (in preformed immune complexes) per 5 × 10^4 glomeruli or per mg of dry organ weight.

Results

Characterization of ATS

In vitro, the ATS bound conspicuously to the mesangial area of the glomeruli in kidney sections from normal rats (indirect immunofluorescence) as well as to margins of thymocytes on thymus sections; the end–point titer was 1:3000 on both organs. No reactivity characteristic of anti-glomerular, basement membrane antibodies was seen [21]. After absorption of undiluted ATS with 1/4 volume of rat thymocytes, binding to the kidney and thymus was abolished even at dilutions as low as 1:10.

The results of ATS absorption by powdered tissue are shown in Table 1. Small amounts of brain or thymus powder eliminated the reactivity of ATS to both the renal glomerulus and thymus. Larger amounts of spleen or kidney powder absorbed the binding of ATS to both organs. Heart or lung powder absorbed minimally, and liver powder did not absorb this activity. The indirect immunofluorescence reaction of a 1:100 dilution of the ATS was abolished by pretreatment of the target sections of kidney and thymus with the monoclonal antibody OX7 (reactive with rat Thy-1.1), but not OX2, which reacts with an unrelated antigen shared by the brain, thymus, and glomeruli. OX7 reacted with glomeruli in an immunofluorescence pattern similar to that of the ATS. Blocking with the amount of OX7 used was minimal when the ATS was diluted only 1:10.

Table 1. Amounts of various organ powders needed to absorb ATS reactivity with glomeruli and thymus

Tissue powder	ATS dilution 1:25		ATS dilution 1:100	
	IF target		IF target	
	Glomerulus	Thymus	Glomerulus	Thymus
Brain	5	25	1	1
Heart	> 50	> 50	50	> 50
Kidney	> 50	> 50	50	25
Liver	> 50	> 50	> 50	> 50
Lung	> 50	> 50	50	50
Spleen	50	> 50	25	10
Thymus	1	25	1	5

The ATS (200 μl) was absorbed with varying amounts (1, 5, 10, 25, or 50 mg) of lyophilized organ powders. The reactivity of absorbed ATS was examined by indirect immunofluorescence on Lewis rat kidney and thymus sections. The values represent the amount of organ powders in mg needed to absorb the reactivity.

Table 2. Glomerular cell counts and immunofluorescence findings

Time after ATS injection[a]	Light microscopy		Immunofluorescence		
	N	No. of Nuclei/Gl	No. of PMN/Gl	Rabbit IgG	Rat C3
1 hr	3	39.8 ± 6.1[b]	2.4 ± 0.4[b]	+++	++
4 hr	3	37.4 ± 6.0[b]	2.8 ± 0.2[b]	++	+
2 days	3	38.9 ± 6.8[b]	0.3 ± 0.1	+	+
4 days	3	54.7 ± 11.5[c]	0.7 ± 0.3	±	–
1 week	3	62.5 ± 9.6[b]	0.1 ± 0.1	–	–
2 weeks	3	64.1 ± 14.2[b]	0.2 ± 0.1	–	–
1 month	3	49.4 ± 8.9	0.1 ± 0.0	–	–
3 months	3	50.6 ± 9.0	0.2 ± 0.1	–	–
Normal rats	4	48.6 ± 7.9	0.2 ± 0.1	–	–

The numbers of nuclei and PMNs were counted individually in over 30 glomeruli (Gl) ranging from 80 to 100 μm in diameter on 2 μm thick sections. The values are expressed as mean ± 1 SD. Student's *t*-test was used for statistical analysis in comparison to normal control rats.

[a] 0.5 ml/100 g body weight, significant

[b] $P < 0.001$

[c] $P < 0.05$

ATS-induced glomerular lesions

In vivo, only trace amounts of rabbit IgG were found in the glomeruli of rats given 0.05 or 0.2 ml/100 g body weight of ATS two days earlier. These rats had no histological abnormalities by light microscopy. 0.5 ml ATS/100 g produced a mild to moderate deposition of rabbit IgG in the mesangium, enlargement of glomerular tufts, and decreased glomerular cellularity at day 2. The mesangial deposits at day 2 were more intense when larger amounts of ATS (2.0 ml/100 g) were given. After seven days, rats receiving 0.5 ml/100 g had no immunofluorescent deposits; however, ballooned glomerular tufts and focal mesangial hypercellularity were conspicuous.

In the sequential histological studies of the ATS-induced glomerular lesions, a small decrease in nuclear counts with karyorrhexis and pyknosis of nuclei and a slight infiltration of PMNs were observed within one hour after ATS administration (0.5 ml/100 g body weight) (Fig. 1A, Table 2). Electron microscopy showed degenerated mesangial cells, leaving epithelial cells or endothelial cells intact, and the mesangium was occasionally infiltrated with PMNs (Fig. 2A, B). Rabbit IgG and rat C3 were found in the mesangium by immunofluorescence microscopy at one hour (Fig. 3A, B). The glomerular lesions examined at four hours resembled those viewed at one hour. By two days, the low-grade PMN infiltration was gone, as were most mesangial cells. Focal areas of the mesangia ballooned and filled with plasma (Fig. 2C), a lesion regarded as mesangiolysis. A small degree of focal mesangial hypercellularity was evident.

By day 4, the glomeruli were enlarged with focal to diffuse mesangial hypercellularity (Fig. 1B, C), increasing the glomerular nuclear counts (Table 2). Occasional PMNs were present, particularly in the areas of mesangial ballooning, that were filled with proteinaceous deposits. Staining for rabbit IgG and rat C3 was weak or negligible (Table 2). By electron microscopy, there were two morphologically different cells in the expanded mesangium; the major cell type was characterized by cells with a large nucleus, single large nucleolus, relatively scant cytoplasm with a smooth cell surface, and scant organelles other than rough endoplasmic reticulum (Fig. 4A). The other cell type had pseudopods on the surface and plentiful cell organelles including phagosomes (Fig. 4B). Both types of cells occupied the mesangiolytic areas and clustered in ballooned regions.

The proliferative lesion persisted at two weeks (Fig. 1D) but had largely subsided by one month and more so at three months, leaving a focal increase in the mesangial matrix and mild sclerotic changes (Fig. 1E). Minimal increases in urinary protein excretion (less than 20 mg/day) occurred during the first 24 hours after ATS injection in some rats, but thereafter no significant proteinuria (more than 5 mg/day) was detected. Creatinine increased slightly but uniformly from a mean of 0.53 mg/dl (baseline) to 0.72 mg/dl at four days after ATS injection ($P < 0.05$). Larger amounts of ATS (0.75 ml or 1.00 ml/100 g body wt) did not increase urinary protein excretion (>20 mg/24 hr) or serum creatinine (>1.0 mg/dl) during the first 14 days after ATS.

Quantitation of glomerular binding of ATS

In paired, label radioisotope studies, ATS administered at a dose of 100 μg/100 g body weight bound to glomeruli after only 15 minutes and the binding was maximum at one hour. The amounts of ATS bound in glomeruli, and livers appear in Table 3. No or minimal ^{125}I-ATS was found in the lungs, brains, hearts, spleens, or thymuses from rats given the ATS 15 minutes, 1, 6, 24 and 96 hours before sacrifice.

The amount of ATS bound to glomeruli at one hour increased linearly with increased dose, as shown in Table 4. The administration of the lesion inducing amount of 0.5 ml ATS/100 g body weight resulted in about 7.6 μg of rabbit IgG binding to 5×10^4 glomeruli. If a total of 3.8×10^4 glomeruli per kidney is assumed [29], then the minimum amount of ATS necessary to produce the mesangial lesions can be calculated as 11.6 μg/2 kidneys.

Quantitation of mesangial function

The uptake of AHGG in various organs was quantitated in ATS- or NRS-treated rats. By sucrose density gradient analysis, the AHGG preparation was slightly larger than human IgM and contained about 10% unaggregated HGG. The uptake of

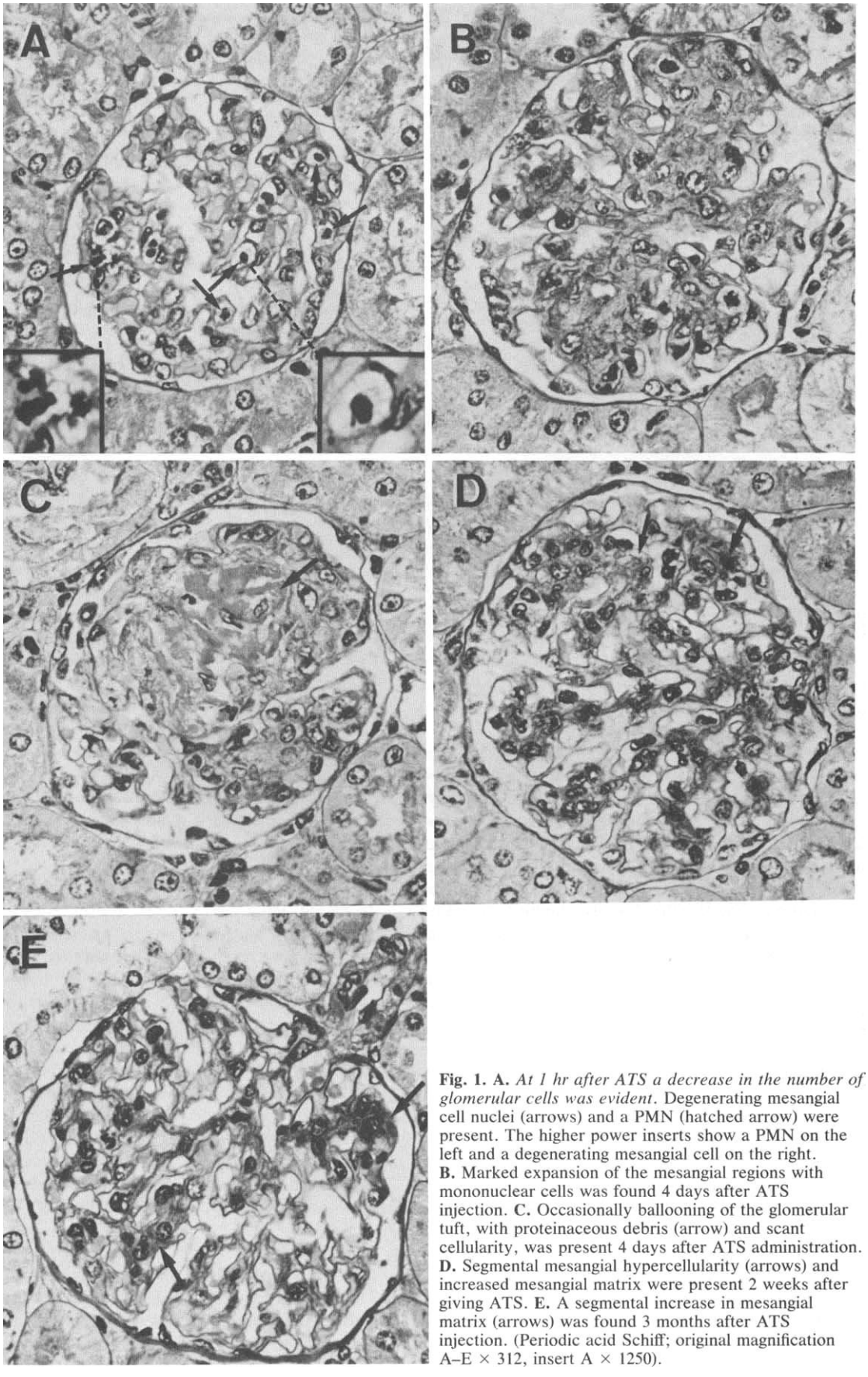

Fig. 1. A. *At 1 hr after ATS a decrease in the number of glomerular cells was evident.* Degenerating mesangial cell nuclei (arrows) and a PMN (hatched arrow) were present. The higher power inserts show a PMN on the left and a degenerating mesangial cell on the right. **B.** Marked expansion of the mesangial regions with mononuclear cells was found 4 days after ATS injection. **C.** Occasionally ballooning of the glomerular tuft, with proteinaceous debris (arrow) and scant cellularity, was present 4 days after ATS administration. **D.** Segmental mesangial hypercellularity (arrows) and increased mesangial matrix were present 2 weeks after giving ATS. **E.** A segmental increase in mesangial matrix (arrows) was found 3 months after ATS injection. (Periodic acid Schiff; original magnification A–E × 312, insert A × 1250).

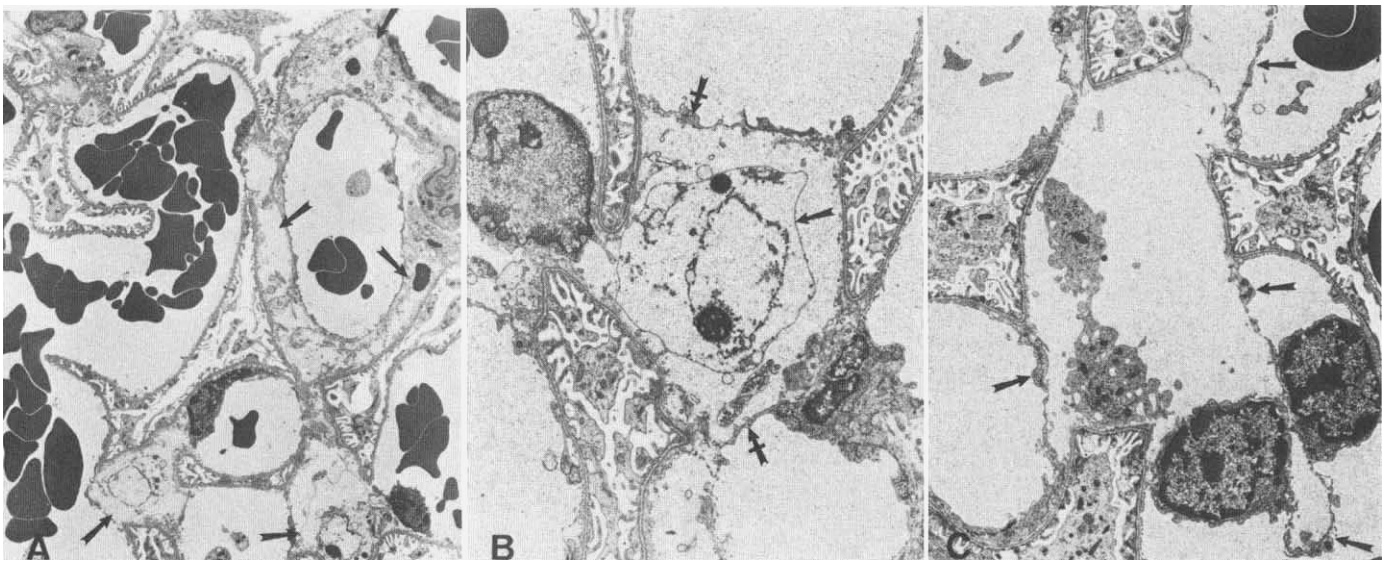

Fig. 2. A. *Diffuse degeneration of mesangial cells (arrows) was observed 1 hr after ATS injection.* PMNs were occasionally present in the mesangial areas. **B.** Mesangial cell destruction (arrow), preserving continuous endothelial cell lining (hatched arrows) and foot processes of epithelial cells, was noted 1 hr after ATS administration. **C.** Mesangial cells had disappeared in areas with residual endothelium (arrows) intact 2 days after ATS injection. (Original magnification: A, × 1800; B, × 9600; C, × 7200).

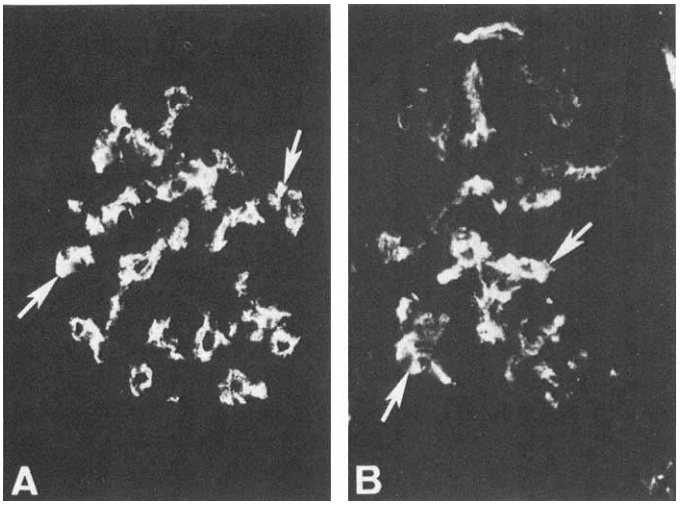

Fig. 3. *Mesangial deposits (arrows) of rabbit IgG* **(A)** *and rat C3* **(B)** *were found in glomeruli 1 hr after ATS injection.* (Original magnification × 400).

AHGG by renal glomeruli in ATS-treated rats increased remarkably at one and four hours in comparison to that in NRS-treated control rats; however, a rapid decrease occurred thereafter, and there was virtually no difference between the ATS- and NRS-treated rats at 24 hours. In both groups uptake of AHGG was similar in livers, lungs, and spleens (Table 5) as well as in hearts and thymuses (data not shown). Large amounts of AHGG accumulated transiently in the liver (Table 5). No significant difference occurred in the clearance of AHGG from the circulations of these two groups (Fig. 5).

In comparison to control animals (Fig. 6A), the immunofluorescent intensity and the distribution of AHGG in the mesangia of ATS-treated animals (Fig. 6B) were greater at one and four hours after the AHGG administration. However, by 24 hours the AHGG deposition was minimal in both groups, which correlated with the quantitative data.

Electron micrographs showed only small electron dense deposits in the mesangial areas of control kidneys obtained one or four hours after the AHGG injection in contrast to extensive deposits in the mesangia after ATS administration. The mesangial cells themselves frequently exhibited hydropic degeneration in the ATS-treated rats. The electron dense deposits in the mesangium were confirmed as human IgG by immunoelectron microscopy (Fig. 6C). The gold–labeled anti-rabbit, IgG antibody used in the immunoelectron microscopy studies did not detect the rabbit IgG of the ATS when administered alone. This may have been due to the lessor amounts of IgG present in the mesangium and the relative insensitivity of the direct rather than indirect techniques used.

The effect of ATS on the glomerular deposition of varying doses of AHGG was examined (Table 6). A significant increase ($P < 0.05$) in the glomerular uptake in ATS-treated rats was found even with 2.5 mg AHGG per 100 g body weight.

The time interval between ATS and AHGG administration as a factor in deposition was also studied by the paired label radioisotope technique. Glomerular uptake of AHGG increased signficantly in all rats given ATS 7, 3, and 1 day or 4 hours before the AHGG administration, with the maximum value at the four hour interval (Table 7). A minimal increase in the glomerular uptake of AHGG was found in rats that were given ATS and AHGG simultaneously.

An increase in the accumulation of ABSA was found in the kidney and glomeruli from the ATS-treated rats given ABSA

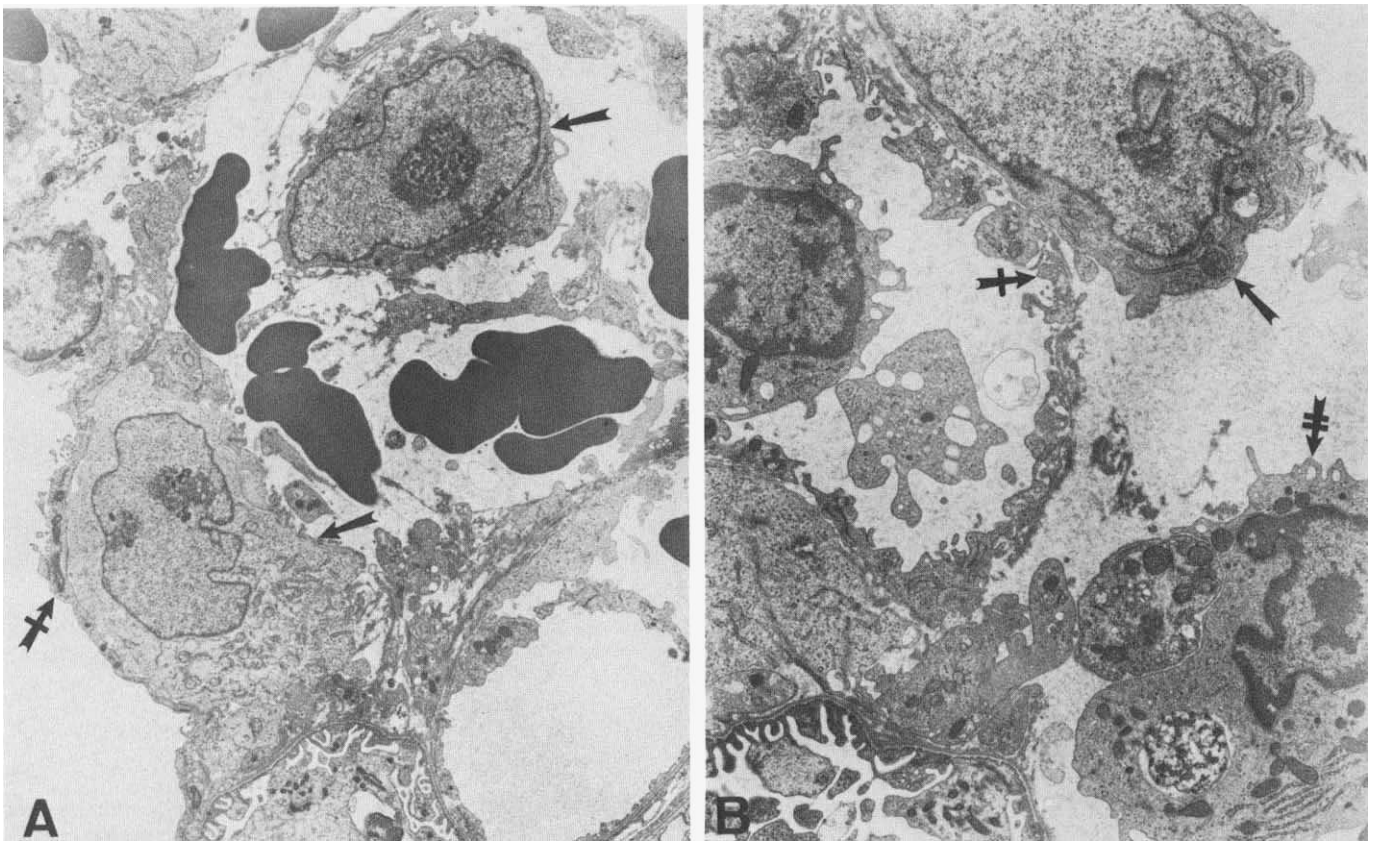

Fig. 4. A. *Two types of mononuclear cells were found in the mesangial regions 4 days after ATS administration.* One type had a large nucleus and large nucleolous (arrows). The endothelium remained well preserved (hatched arrow), maintaining capillary integrity. **B.** The other cell type had pseudopods (double hatched arrow) and many cell organellas, and was presumed to be a monocyte/macrophage. The first cell type (arrow) is shown for contrast. The endothelium (hatched arrow) was intact. (Original magnification: A, × 3000; B, ×4800).

Table 3. Specific binding of ^{125}I-ATS

Tissue	Time after ATS administration uptake as μg/g dry weight				
	15 min	1 hr	6 hrs	24 hrs	96 hrs
Glomeruli	7.8 ± 0.9	11.6 ± 0.9	8.0 ± 0.5	3.4 ± 0.5	2.5 ± 0.7
Liver	0.0 ± 0.0	0.1 ± 0.0	0.4 ± 0.1	0.3 ± 0.0	0.1 ± 0.0

Rats received 100 μg each of ^{125}I-ATS and ^{131}I-NRS gammaglobulin fractions intravenously and were sacrificed at the time intervals shown in the Table. The specific uptake in the organs was calculated as μg protein per gram weight of dried organ. The values are mean ± 1 SD (N = 4 per time point). No or minimal specific uptake was found in lungs, brains, hearts, spleens, or thymuses.

Table 4. Binding of rabbit IgG to glomeruli one hour after intravenous administration of large amounts of ATS

Body weight *ml ATS/100 g*	Uptake in 5 × 10^4 glomeruli		Mesangial cell degeneration
	Rat 1 μg	Rat 2 μg	
0.05	2.8	3.9	no
0.2	4.0	4.9	no
0.5	7.5	7.8	yes
2.0	11.6	15.5	yes
5.0	19.4	24.3	yes

The values are μg rabbit IgG bound to 5 × 10^4 glomeruli prepared from the two individual rats used for each ATS dose.

four or 24 hours before sacrifice. In this experiment, the difference in glomerular uptake between control and ATS-treated rats remained significant even after 24 hours, whereas the other organs took up equivalent amounts in both groups (Table 8). The clearance of ABSA from the circulation was also the same in both groups.

The proportion of radiolabeled HGG precipitated by addition of polyethylene glycol to the preformed immune complex preparation was 88.3%, whereas less than 5% of control monomeric HGG was precipitated by the procedure. More of the preformed immune complexes accumulated in glomeruli from ATS-treated rats than from controls when examined four hours after the injection, and the residual amount of these complexes in the glomeruli at 24 hours was less in treated than in control rats (Table 8). There were no significant differences in other organs between control and ATS-treated rats. There was no significant difference in the clearance of the preformed immune complexes from the circulation between two groups.

Table 5. Time course of AHGG uptake in glomeruli and organs

	μg/mg Dry weight, $\mu g/5 \times 10^4$ *glomeruli*		
	1 hr	4 hr	24 hr
Glomeruli			
ATS	96.5 ± 19.1[a]	24.4 ± 3.1[a]	1.1 ± 0.2
	(453.1 ± 28.3[a])	(126.7 ± 19.0[b])	(6.3 ± 1.1)
control	4.1 ± 1.4	2.2 ± 0.5	0.9 ± 0.4
	(11.2 ± 3.0)	(9.1 ± 1.1)	(5.0 ± 1.0)
Liver			
ATS	12.1 ± 3.7	6.8 ± 1.8	0.2 ± 0.1
control	13.4 ± 4.4	6.1 ± 0.3	0.2 ± 0.1
Lungs			
ATS	2.1 ± 0.4	0.7 ± 0.1	0.1 ± 0.0
control	2.3 ± 0.8	0.9 ± 0.2	0.1 ± 0.0
Spleen			
ATS	12.8 ± 1.3	2.4 ± 0.3	0.3 ± 0.1
control	12.2 ± 0.7	2.7 ± 0.5	0.3 ± 0.0

The rats pretreated with ATS or control NRS 24 hr earlier were injected with ^{125}I-AHGG (50 mg/100 g body wt) and sacrificed at the intervals shown. The uptake of AHGG (μg) per mg dry weight of organ is expressed as mean ± 1 SD. The uptake per 5×10^4 glomeruli before drying is given in parentheses.

[a] Statistically significant ($P < 0.001$) between control ($N = 4$) and ATS-treated rats ($N = 4$)

[b] $P < 0.05$

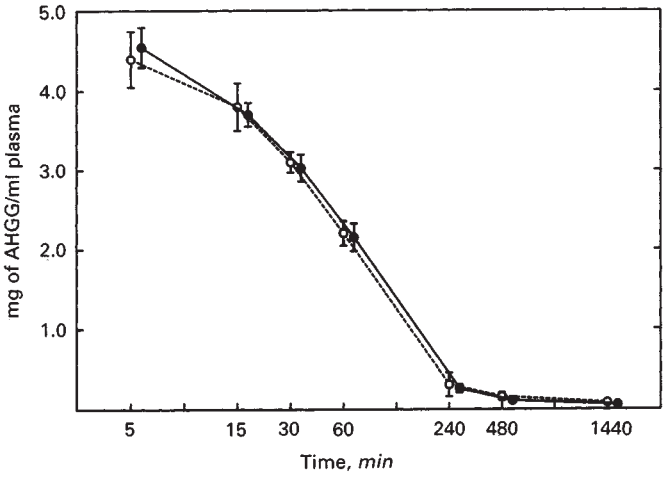

Fig. 5. *Clearance of ^{125}I-AHGG from the circulation in control (open circles) and ATS-treated rats (closed circles).* The bars depict 1 standard deviation.

Discussion

This model constitutes a prototype of a new class of nephritogenic immunologic reaction in which antigens believed to exist naturally, or perhaps trapped, on the surface of glomerular (or tubular) cells can serve as targets for nephritogenic immune attack. The immunopathogenesis of glomerular injury, until recently, has been considered to be secondary to immune deposit formation by one of several mechanisms [30], with subsequent immune mediator–induced inflammation, leading to nonspecific damage to surrounding structures. The model described herein results in specific and selective injury in an anatomic structure containing multiple cell types. Other potential glomerular cell surface antigens being considered candidates in this type of reaction would be angiotensin converting enzymes on the surface of endothelial cells [31, 32], the epithelial cell, foot process antigen of spontaneous glomerulonephritis in rabbits [33], and the Heymann's nephritis gp330 reactant identified on epithelial cells, as well as on the brush border area of the renal proximal, tubule epithelial cell [34–36]. The latter antigen system has been used also as a target for damage to proximal tubular cells [37, 38].

In the ATS-induced glomerular lesion, the relevant target is the mesangial cell and the selective damage of mesangial cells was apparent in the electron micrographs. Specific binding of intravenously administered ATS to mesangial cell surface has been found [16]. The early decrease in mean glomerular cell number from 48.6 to 37.4 to 39.8 per glomerulus within two days could be interpreted to mean that 72.4 to 92.2% of the mesangial cells, estimated to comprise about 25% of glomerular cells [39], were damaged by the ATS treatment.

The antigen recognized with ATS is probably Thy-1 on the mesangial cells because the reactivity of ATS with glomeruli was completely absorbed with thymocyte or brain powder and largely inhibited by monoclonal anti-Thy-1.1 antibody, but not by a monoclonal antibody reactive with another antigen shared by the brain and thymus. In addition, a Thy-1-like antigen was previously localized in glomeruli by immunofluorescence [12–14] and on the surface of mesangial cells by immunoelectron microscopy [15, 16]. The Thy-1 antigen is one of the lymphocyte differentiation antigens [40], and in the rat is found on thymocytes and a portion of nucleated, bone marrow cells [41]. Most peripheral T lymphocytes are Thy-1 negative in the rat, in contrast to their positive state in mice [42]. The lack of antigen on circulating cells may facilitate its ability to reach the mesangial area.

Mesangial cell death resulted from the binding of about 11.6 μg antibody per 7.6×10^4 glomeruli (the assumed glomerular content of two rat kidneys [29] one hour after ATS administration. This led to the initial lytic and subsequent proliferative/infiltrative lesions in the mesangium. In quantitative terms, the amount of glomerular–bound antibody required to cause the lesion was less than that usually necessary to induce glomerular injury in immune deposit forms of experimental rat nephritis. For example, about 150 μg of antibodies/2 kidneys are required to produce heterologous phase anti-glomerular, basement membrane nephritis [43], and a similar amount of anti-Con A antibody is needed in the Con A planted antigen model of glomerular injury [44]. In contrast to these models in which immune deposits and mediator activation damage surrounding glomerular tissues, the anti-Thy-1 antibody reacts with the surfaces of mesangial cells, presumably damaging them directly. Accordingly, it is not surprising that the direct effect described here should require less antibody to produce its focused injury. Preliminary studies suggest that the acute antibody–induced, mesangial cell injury depends on complement and can be suppressed by decomplementation with cobra venom factor, but not leukocyte depletion using irradiation or anti-PMN serum [18, 45, 46]. In addition, late components of complement (C5, C6, C7, C9, C5b-9 complex) were defined by indirect immunofluorescence in the mesangium of the kidneys which were obtained one hour after the ATS injection [46]. The localization of these components was closely associated with

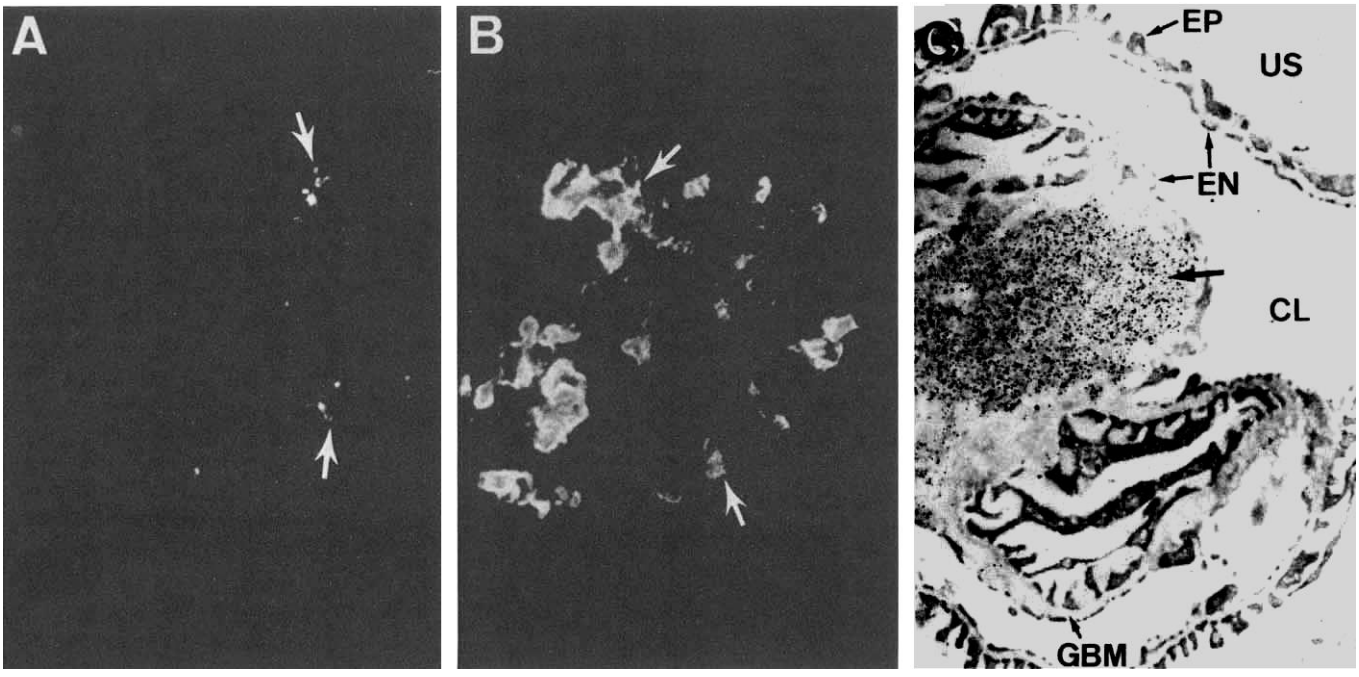

Fig. 6. A. *Small, focal deposits of human IgG were found in the mesangium of a control rat given AHGG 1 hour earlier.* **B.** In contrast, massive, diffuse deposits of human IgG were present in the mesangium of a rat given AHGG that had received ATS 4 hr earlier. **C.** The colloidal gold technique was used to identify AHGG (human gammaglobulin) in the glomeruli at the electron microscopic level. The black colloidal gold particles (arrows) are packed in the mesangial areas corresponding with electron dense deposits. Abbreviations are: EP, epithelial cell; US, urinary space; EN, endothelium; CL, capillary lumen; GBM, glomerular basement membrane. (Original magnification A, B × 400; C × 14,000).

Table 6. Dose response of AHGG uptake in glomeruli and liver

^{125}I-AHGG *mg/100 g body wt*	Glomeruli *μg/5 × 10⁴*	Glomeruli *μg/mg Dry weight*	Liver *μg/mg Dry weight*
50 mg			
ATS	453.1 ± 28.3[a]	96.5 ± 19.1[a]	12.1 ± 3.7
control	11.2 ± 3.0	4.1 ± 1.4	13.4 ± 4.4
25			
ATS	185.0 ± 42.5[a]	35.0 ± 5.9[a]	12.7 ± 0.9
control	4.6 ± 2.5	0.9 ± 0.4	12.3 ± 1.9
10			
ATS	14.1 ± 1.6[b]	3.3 ± 0.4[b]	8.2 ± 1.5
control	6.0 ± 3.7	1.3 ± 0.7	9.2 ± 1.3
2.5			
ATS	1.5 ± 0.4[b]	0.3 ± 0.1[b]	2.3 ± 0.6
control	0.7 ± 0.2	0.1 ± 0.0	2.4 ± 1.1

All groups (four rats each) pretreated with ATS or control NRS 24 hours earlier were sacrificed one hour after the administration of ^{125}I-AHGG for measurement of the uptake by paired label method. The uptake of AHGG is expressed both in microgram per 5 × 10^4 glomeruli and per mg dry weight of glomeruli or liver. Data are mean ± 1 SD.

[a] $P < 0.001$

[b] $P < 0.05$

Table 7. Time of ATS administration versus uptake of AHGG

ATS	Glomeruli *μg/5 × 10⁴*	Glomeruli *μg/mg Dry weight*	Liver *μg/mg Dry weight*
−7 days	74.5 ± 16.4[a]	9.7 ± 2.4[a]	8.6 ± 0.3
−3 days	177.0 ± 40.9[a]	25.6 ± 4.3[a]	7.8 ± 0.6
−1 day	182.9 ± 50.8[a]	24.7 ± 6.3[a]	9.6 ± 0.3
−4 hours	643.4 ± 83.7[a]	78.2 ± 14.5[a]	10.1 ± 0.9
0	40.8 ± 28.8	5.5 ± 3.7	9.4 ± 1.6
control	13.5 ± 3.2	2.1 ± 0.3	9.7 ± 0.3

All groups (three rats each) given ATS at the times specified or left untreated (control) were sacrificed one hour after the ^{125}I-AHGG injection (50 mg/100 g body wt). Both the uptake of AHGG (μg) per 5 × 10^4 glomeruli and per mg dry weight of glomeruli, as well as liver uptake, are represented as mean ± 1 SD.

[a] $P < 0.001$

that of rabbit IgG and rat C3. Similar glomerular lesions to the ATS-induced lesions were demonstrated in rats by intravenous administration of complement-fixing, monoclonal anti-thymocyte antibody but not by non-complement-fixing antibody [19]. These results suggest that the membrane attack complex [47], formed on the mesangial cell membrane, plays a role in the cell injury and the following proliferative response.

The role of mesangial cells in the accumulation of aggregates within glomeruli is not completely understood, and the degree of phagocytic activity of these cells is not quantified [4, 48]. To assess the effect of selective mesangial cell damage on the mesangium's uptake of macromolecular substances, several large aggregates were prepared and given to rats that had received ATS i.v. Immunofluorescence studies and quantitative, paired labeled experiments both showed a remarkable increase in the glomerular uptake of these aggregates in ATS-treated cells. Immunoelectron microscopic studies confirmed that the massive deposition of aggregates was exclusively in the

Table 8. Uptake of ABSA and preformed immune complexes four or 24 hours after administration

	Uptake of ABSA μg/mg Dry weight		Uptake of immune complexes μg/mg Dry weight	
	4 hr	24 hr	4 hr	24 hr
Glomeruli				
ATS	81.4 ± 5.9[a]	17.7 ± 6.9[b]	36.4 ± 5.1[b]	0.6 ± 0.4[b]
	(333.2 ± 45.2[a])	(78.0 ± 29.0[b])	(188.0 ± 22.0[b])	(4.6 ± 2.1)
Control	7.3 ± 2.7	0.8 ± 0.3	11.3 ± 2.1	2.7 ± 1.6
	(22.9 ± 6.7)	(3.5 ± 1.0)	(45.9 ± 13.7)	(10.1 ± 5.0)
Liver				
ATS	0.4 ± 0.0	0.0 ± 0.0	11.7 ± 1.4	1.4 ± 0.7
Control	0.4 ± 0.0	0.0 ± 0.0	13.7 ± 1.8	1.4 ± 0.5
Spleen				
ATS	3.5 ± 0.5	1.3 ± 0.2	2.8 ± 0.6	1.5 ± 0.1
Control	3.0 ± 0.6	1.1 ± 0.2	3.0 ± 0.5	1.6 ± 0.2

The rats pretreated with ATS or NRS (control) 24 hours earlier received ^{125}I-ABSA (25 mg/100 g body wt) or preformed immune complexes (40 mg ^{125}I-HGG/100 g body wt). The data (mean ± 1 SD, N = 4 or 5) are presented as μg uptake of ABSA or HGG in preformed immune complexes per mg dry weight of organ. The uptake per 5×10^4 glomeruli before drying is given in parentheses.

[a] $P < 0.001$

[b] $P < 0.05$

mesangial area. The cause of the increased uptake in the glomerular mesangium was intraglomerular factors, since there were no differences in the clearance of aggregates from the circulation or uptake in the reticuloendothelial system, such as liver or spleen, between control and ATS-treated rats. The increased mesangial accumulation of macromolecular aggregate must be the consequence of an alteration in the balance between ingress (uptake) and egress (disposal). The striking increase in the one hour mesangial accumulation of AHGG in rats treated four hours to seven days earlier with ATS must reflect either or both facilitated transportation of AHGG from the circulation to the mesangium (ingress) and a reduced egress by transport via the mesangial channels or back to the glomerular capillary as well as any contribution by mesangial cell processing. The current study cannot differentiate between those possibilities. However, it does appear that mesangial uptake of macromolecules increases remarkably when the mesangial cell is injured or when inflammatory reaction, including complement activation, focuses on the mesangium.

The rapid removal of the large amount of AHGG (and other macromolecular markers) initially trapped suggests that mesangial cell processing, which was no longer possible during the lytic phase of injury, can occur without active participation of the mesangial cell. Actually, egress may have been facilitated by the disruption of the normal mesangial architecture, allowing more rapid movement of the macromolecules into the vascular stalk or elsewhere. Another possibility is that the immunoglobulin containing aggregates, AHGG, and immune complexes were acted on by the few PMNs that accumulated in the glomeruli or by monocytes in the mesangial areas [49, 50], as well as attracted there, as reported, after injection of immune complexes into mice [48]. In support of this idea, more ABSA persisted in the mesangium 24 hours after its introduction when compared with AHGG and preformed immune complexes. This may be attributed to less immunoreactivity of ABSA than AHGG or immune complexes in terms of Fc or possibly complement receptor interactions with monocytes or PMNs. However, the number of infiltrating cells in a glomerulus is small during the early hours of the ATS-induced lesion; therefore, their contribution to the clearance of mesangial deposits may not be marked.

The origin of the mesangial–proliferative lesions that followed two to four days after ATS administration remains to be defined. The electron microscopic studies suggest that the cells are composed of both mesangial cells and infiltrating leukocytes. In preliminary studies, leukocyte depletion using irradiation only diminished somewhat but did not prevent the hypercellular lesion [18], suggesting that the majority of cells were actually mesangial in nature. The lesion resolved with residual increase in mesangial matrix and focal sclerosis, a response also presumed to reflect the mesangial cell response to the initial destructive phase of injury.

Mesangial damage consisting of focal ballooning and hypercellularity has been observed in rats and rabbits given Habu (*Trimeresurus flavoviridis*) venom i.v. [10, 11]. The Habu venom lesion is much less extensive in rats than the ATS-induced lesion, and the mesangial cells tend to be preserved intact or unaffected. The Habu venom–induced lesion is considered to be generated, at least in part, by enzymatic activity of the venom on mesangial matrix. Venom-induced platelet aggregates may also contribute to endothelial damage, and coagulation may also play a role [11].

Of interest, the striking glomerular lesion associated with the ATS-induced mesangial cell injury, lysis, and proliferation/infiltration caused only minimal changes in excretion of protein in the urine and serum creatinine elevation. The same antibody given to Munich–Wistar rats did cause greater proteinuria (data not shown), suggesting that strains may differ in the severity of pathophysiologic manifestations. Since the glomerular capillary wall serves as the major filtration barrier, it is perhaps not surprising that selective mesangial injury would not be associated with marked changes in glomerular solute or macromolecular clearances. Recently, proteinuria was produced in Wistar rats using a monoclonal anti-Thy-1.1 antibody which reacted along the glomerular basement membrane as well as in the mesangium [19, 20].

The identification of an antibody and presumably complement–associated mechanism of selective mesangial cell injury adds an important new dimension to the current concepts of immunopathologic mechanisms of renal injury. Now direct and selective mechanisms of renal cell injury must be considered alongside the well established but less selective forms of immune glomerular insult generated by various modes of antibody accumulation in the glomerulus, followed by humoral and cellular mediator activation with indiscriminate injury to adjacent structures. The selective mesangial injury was associated with marked changes in glomerular handling of macromolecular aggregates; however, in spite of severe histologic changes, little effect was seen on glomerular clearance of small or large molecules defined by serum creatinine and urinary protein measurements. This selective effect on function of various glomerular structures is not usually achieved with less specific forms of immune insult. The human counterparts of the new immunologic mechanism remain to be defined. The report of anti-mesangial antibody activity in immunoglobulin eluted from

the kidneys of a patient with mesangial IgA nephropathy is of interest in this regard [2]. Since antibody–induced, mesangial cell injury does alter mesangial handing of macromolecules, direct or indirect immunologic mesangial damage in the form of antibody and/or complement activation with inflammation could further impair mesangial function and compound mesangial immune–complex deposition.

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